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ETIOLOGY AND RAPID DIAGNOSIS OF HUMAN VIRAL GASTROENTERITIS

Annual Report

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This project is designed to assess the etiology and establish the rapid diagnosis of human viral gastroenteritis. During the contract year for this report, we have continued to demonstrate the involvement of astroviruses in gastroenteritis. In a previously reported Thai study, we have now determined that the clinical symptoms of patients with astrovirus infections were similar to the symptoms of those with rotavirus infection. Additional studies in Guatemala (5,234 samples) and Australia (1,343 samples) have confirmed the importance of astroviruses in gastroenteritis in comparison to other virus infections (rotaviruses and enteric adenoviruses). During this contract year we also developed monoclonal antibodies to calicivirus and we are evaluating these antibodies for use as diagnostic reagents. Ongoing collaborative epidemiological studies have continued to be done with the U.S. military in overseas populations, utilizing the immunodiagnostic techniques that we have developed for gastroenteritis viruses.

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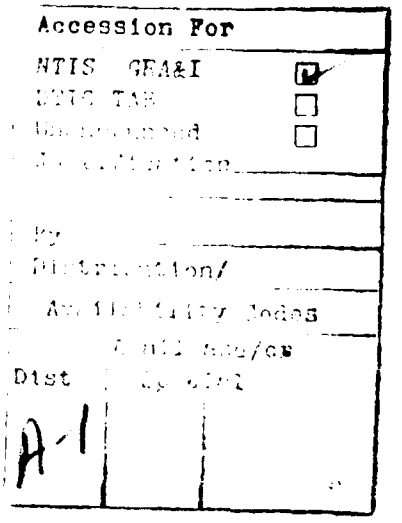
SUMMARY

This project is designed to assess the etiology and establish the rapid diagnosis of human viral gastroenteritis. Of primary importance is the development and utilization of immunoassays to detect various viruses, with preparation and use of monoclonal antibody reagents where possible. Following the successful development of diagnostic assays, the medical and epidemiological importance of these viruses is being ascertained. During the contract year for this report, we have continued to demonstrate the involvement of astroviruses in gastroenteritis. In a previously reported Thai study, we have now determined that the clinical symptoms of patients with astrovirus infections were similar to the symptoms of those with rotavirus infection. Additional studies in Guatemala (5,234 samples) and Australia (1,343 samples) have confirmed the importance of astroviruses in gastroenteritis in comparison to other virus infections (rotaviruses and enteric adenoviruses). The large number of samples examined was possible because of the monoclonal antibody immunoassays we have developed to rotaviruses and enteric adenoviruses as well as to astroviruses. During this contract year we also developed monoclonal antibodies to calicivirus and we are evaluating these antibodies for use as diagnostic reagents. Ongoing collaborative epidemiological studies have continued to be done with the U.S. military in overseas populations, utilizing the immunodiagnostic techniques that we have developed for gastroenteritis viruses.



FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

CONTENTS

BACKGROUND INFORMATION ON VIRAL GASTROENTERITIS

Acute viral gastroenteritis is an extremely common illness that affects all age groups and occurs in both epidemic and endemic forms (1). It is second in frequency only to the common cold among illnesses affecting United States families under epidemiological surveillance. It is also responsible for some of the common travelers' diarrhea encountered in Latin America, Africa, and Asia. The illness varies in its clinical presentation, but in general it begins with an explosive onset, and consists of varying disabling combinations of diarrhea, nausea, vomiting, low grade fever, abdominal cramps, headache, anorexia, myalgia, and malaise. It can be severe, indeed fatal, in the elderly, infant, debilitated, or malnourished patient.

Viral gastroenteritis occurs primarily in two epidemiologically distinct clinical forms (1). One entity is characteristically epidemic and is responsible for family and community-wide outbreaks of gastroenteritis among older children and adults. In recent years, one agent, Norwalk virus, has been shown to be responsible for about 40 percent of these disease outbreaks in the United States. Other Norwalk-like viruses have also been discovered such as Hawaii virus and Snow Mountain virus, and although they have not been well studied epidemiologically, they are likely to be responsible for many more epidemic cases of this illness.

The second clinical entity is usually sporadic, occasionally epidemic, and occurs predominantly in infants and young children (1). However, as noted below it can occur in adults. This form of illness typically produces severe diarrhea that commonly lasts for five to eight days and is usually accompanied by fever and vomiting. Rotavirus is responsible for nearly one half of the cases of this clinical entity requiring hospitalization. Although the major target of rotavirus is the very young, it can produce surprisingly severe clinical disease in adults (1,2).

Breakthroughs in determining the medical importance of Norwalk virus and rotavirus occurred primarily because of the development of immunoassay techniques to recognize these viruses in stool samples and to measure antibodies to them in infected individuals. For Norwalk virus, these assays are currently available in only a few research laboratories (3,4) including that of the principal investigator. This is because the procedure requires use of precious limited human volunteer materials (stools and sera). The assay has more recently been made more efficient in detecting Norwalk virus antigen in stools through the use of an enzyme-linked immunoassay (EIA) instead of a radioimmunoassay (RIA) (5). Together with our collaborators, our use of these immunoassays has shown a major role for this virus in producing in the U.S. clam and oyster associated gastroenteritis, as well as some cases of

travelers' diarrhea in Mexico and Thailand (6-8).

As for rotavirus, use of immunoassay techniques to detect the virus is now common and is employed routinely in many clinical diagnostic laboratories (9). More recently, a monoclonal antibody based EIA that we developed for detection of rotavirus (10) has been shown to be more sensitive and specific than polyclonal antibody tests and has eliminated specificity problems with stool samples from young infants. We have used rotavirus immunoassays to establish the role of rotavirus in several nations around the world, including travelers' diarrhea experienced by U.S. military populations overseas (7, 11-15).

The roles of other enteric viruses in gastroenteritis are incompletely understood, and because of the medical importance of infectious diarrhea, there is clearly a major need to establish the significance of different viruses that may be involved. Comparative studies on their occurrence, however, have been infrequent and usually limited to electron microscopy (15-17). The major obstacle in evaluating the relative importance of the non-rotavirus and non-Norwalk virus enteric viruses as causative agents of gastroenteritis has been the lack of convenient methods for their diagnosis. In addition, for appropriate treatment and control measures to be initiated, rapid as well as convenient methods are required, but have been also unavailable for most of these gastroenteritis viruses. Further, many of these viruses are difficult to cultivate or have not been cultivated in cell culture, which has inhibited characterization studies.

Among these agents, the evidence currently seems strongest that enteric adenoviruses and now astroviruses are medically important pathogens like rotavirus and Norwalk virus. The enteric adenoviruses differ from the well characterized conventional serotypes of adenoviruses which are propagated in standard tissue cultures and are not commonly associated with gastroenteritis. These adenoviruses can not be recognized specifically as enteric types by electron microscopy in stools and can be cultivated only inefficiently in an adenovirus transformed cell line, Graham 293 (18). Two enteric serotypes (types 40 and 41) have been identified and in a limited number of studies performed to date, have been associated with gastroenteritis in infants and young children and much less commonly found in asymptomatic children (1,19). The potential role of enteric adenoviruses in travelers' diarrhea or in disease in adults has been little studied. Convenient and specific immunoassays to detect enteric adenoviruses have been only recently developed and now permit an understanding of their epidemiology as has already occurred with the use of immunoassays to study rotavirus and Norwalk virus. Four years ago we prepared monoclonal antibodies specific for adenovirus types 40 and 41. These antibodies were characterized and used in an EIA format to detect the enteric adenoviruses in known positive diarrheal stool specimens with 95 to 98 percent sensitivity and specificity

(20,21). In 1988 we reported that two percent of acute diarrheal episodes among Thai children were due to enteric adenoviruses using the EIA procedure (22). We are using our enteric adenovirus monoclonal antibody EIA to assess the epidemiology of this infection in other populations on a continuing basis.

Astroviruses are small (27-35mm in diameter) round viruses and have been identified by electron microscopy in the stools of some patients with gastroenteritis (1,23). Astroviruses also have been shown to be cultivatable in cell culture (24,25). However, simple diagnostic procedures have not been widely available prior to our work. Thus, the extent of the role of astroviruses in human diarrheal disease is not known. In 1988, we published our confirmation of in vitro cultivation of human astroviruses (26). This permitted us to purify sufficient viral antigen to prepare monoclonal antibodies reactive against a common antigen shared by multiple astrovirus serotypes (26), which offered the practical possibility for developing immunoassays to assess the medical importance of astroviruses in human viral gastroenteritis. Such an EIA test for the detection of astroviruses in human stools was developed by us and was published in the Journal of Infectious Diseases (27). Since that time we have been using the assay extensively in ongoing epidemiological studies.

Caliciviruses have also been associated with diarrheal disease in humans (1,28). These agents are currently detected mostly by electron microscopy and more convenient assays for their detection are needed so that their epidemiology can be studied. Norwalk virus possesses a single structural protein, characteristic of a calicivirus (29), and the two agents are of similar size and general shape (albeit, differing somewhat in virion surface structure). Thus, the possibility of relatedness between these two enteric viruses exists and was studied by us two years ago. We demonstrated that antigenic characteristics are shared between calicivirus and Norwalk virus based on our detection of seroconversions to Norwalk virus in patients experiencing gastroenteritis due to a strain of calicivirus (30, 31). These two agents, therefore, may belong to the same family of viruses, as also may Snow Mountain agent for which we have also found seroconversions to Norwalk virus in some affected patients (32). These serological cross-reactions demonstrate the need for convenient viral antigen specific detection methods for calicivirus such as we previously developed for Norwalk virus.

ASTROVIRUS ETIOLOGY OF VIRAL GASTROENTERITIS

Prior to our studies, astroviruses had been associated with gastroenteritis but had not been directly shown to be etiological agents in a controlled study. In our annual report for 1990 we described two studies in Thailand which indicated a causative role for astroviruses. To further assess the role and medical impor-

tance of astroviruses in gastroenteritis in these studies, we compared the clinical symptoms found in gastroenteritis patients infected only with astroviruses to those infected only with rotaviruses; patients with other bacterial, viral or parasitic infections were excluded. The results are given in Table 1. The frequency of clinical findings detected with each of these two virus infections was similar, and no significant differences were found in any of the categories evaluated. Dehydration $\geq$ 5 percent was more common with rotavirus infections but was not significant ($P < 0.08$).

The rate of hospitalization was low in both rotavirus and astrovirus infections, but there was a slightly higher percentage of hospitalized children who had rotavirus infection alone, 3.4 percent (6 of 175) compared to 2.2 percent (1 of 44) for those with astrovirus infection alone. This may have been due to the higher rate of rotavirus infected patients showing dehydration $\geq$ 5 percent (27 of 175 compared to 2 of 44), although all of the remaining patients with astrovirus infections showed a lower level of dehydration (<5 percent) as did the remainder of those infected with rotaviruses. The results of these studies are now in press (New England Journal of Medicine, reference 33).

Table 1. Clinical findings associated with astrovirus and rotavirus gastroenteritis

Clinical finding	% showing clinical finding	
	Astrovirus infection[a] n = 44	Rotavirus infection[a] n = 175
Watery stools	61	67
Loose stools	41	35
Mucoid stools	55	51
Bloody stools	7	6
Nausea	71	88
Abdominal pain	58	63
Vomiting	61	67
Fever	80	83
Dehydration $\geq$ 5%	5	15

[a]Includes only those samples where no bacterial, parasitic, or other viral pathogens were detected.

In addition to correlating clinical findings with astrovirus infection in this population, we are also testing for sets of viruses, bacteria, and parasites that discriminate between patients with and without gastroenteritis by means of the multivariate method, stepwise logistic regression. A summary of the major findings to date are shown in Table 2. The greater the chi-square

improvement value, the greater the independent association of a given pathogen with disease status. It can be seen that astroviruses were ranked quite high compared to some of the more widely recognized pathogens.

Table 2. Ranking of pathogens as discriminators of disease by logistic regression analysis.

Rank	Pathogen	Improvement Chi-square
1	Rotavirus	272.6
2	Shigella spp.	223.4
3	Enteropathogenic E. Coli	40.1
4	Astrovirus	31.5
5	Crytosporidium spp.	15.7
6	Salmonella spp.	13.0
7	Campylobacter jejuni	10.5

During this contract year, additional studies on astroviruses have been done in a variety of populations. The data given in Table 3 summarizes the major findings to date for astrovirus gastroenteritis in pediatric populations. All show a major medical role for astroviruses.

Table 3. Incidence of astrovirus diarrhea in various populations and comparison to rotavirus and enteric adenovirus infection.

Geographic Area	No. Tested	Diarrheic (+ or -)	% virus positive		
			Rotavirus	Astrovirus	Enteric Adenovirus
Thailand	1,691	+	19.0	8.2	2.6
(2 studies)	1,459	-	1.0	2.0	0.5
Atlanta,	524	+	nt	4.0	nt
Georgia[a]	105	-	nt	1.0	nt
Australia (Aborigines)	1,343	+	6.8	5.5	1.3
Guatemala	5,234	+	3.0	3.7	0.2

[a] In collaboration with R.I. Glass, CDC, Atlanta, submitted for publication.

CALICIVIRUS MONOCLONAL ANTIBODIES

To maximize the possibility of obtaining monoclonal antibodies with the limited amount of virus we have available, we initially examined methods for purifying virus from stools using feline calicivirus seeded stools as a model, and applied the results of these model studies to purification of calicivirus antigen from human stool samples. These samples were known to contain calicivirus by IEM. Fractions were inoculated into mice for production of antisera and eventual use in hybridomas. By ELISA techniques we determined that virus-specific antibody was being produced by the mice, and fusions for hybridoma production were done. Clones producing antibody to calicivirus, as determined by ELISA, have been obtained and we are in the process of evaluating them. The utility of these monoclonal antibodies in diagnostic tests remains to be determined.

We are also trying to prepare antibodies to calicivirus antigen in rabbits. This would provide us with sera for use in immunoassays along with our monoclonal antibodies. The antigen used as inoculum was purified from stool material, and because we have a limited quantity of material to work with, a recently introduced adjuvant (Titermax, CytRx Corp, Norcross, GA), which is purported to be superior to Freund's adjuvant, was used.

SNOW MOUNTAIN VIRUS MONOCLONAL ANTIBODIES

Sometime ago, we had received human peripheral blood lymphocytes from volunteer patients who had received Snow Mountain Virus (courtesy of Dr. R. Dolin, University of Rochester). We had transformed these cells with Epstein-Barr virus for use in future studies. To look at the possibility of preparing human monoclonal antibodies with these and other cells, the transformed cells were fused with human x mouse heterohybridomas. These will hopefully result in hybridomas which produce human antibody specific for Snow Mountain virus. We are in the process of preparing and screening such hybridomas.

ANTIIDIOTYPIC ANTIBODIES AS SURROGATE ANTIGENS

To perform diagnostic assays designed to detect viral antigen, a positive control antigen is necessary. This can be a problem if virus cannot be cultivated and there is a limited amount of clinical material available for this purpose. It has been shown for several viruses, such as hepatitis B virus, Herpes simplex virus, and Newcastle Disease virus among others, that anti-idiotypic antibodies to viral antibodies, which have been likened to an "internal image" of an antigen, can be used as a substitute for the antigen itself. To demonstrate feasibility of this

approach, we are working with astrovirus as a model for the non-cultivatable viruses (calicivirus, Norwalk virus, SRVs). We have now generated polyclonal anti-idiotypic antibody to astrovirus monoclonal antibody, which demonstrates that this approach is practical and sound. We are also preparing monoclonal anti-idiotypic antibodies to astrovirus monoclonal antibody as a model system for other viruses. To do this, BALB/c mice were inoculated with our BALB/c mouse-derived monoclonal antibody to astrovirus (purified IgG, emulsified in Freund's incomplete adjuvant, given i.p at 75 ug IgG/mouse). Five additional inoculations were given at two week intervals and mice were bled and tested for anti-idiotypic antibody. For this test, the serum was substituted for viral antigen in our astrovirus EIA. Pre-immune serum was used as a control. The positive reactions obtained indicate that monoclonal antibodies can be derived from these reactive mice, and we are now starting to evaluate these materials in monoclonal antibody based assays for detection of astrovirus. If we can successfully obtain a monoclonal antibody based immunoassay for astrovirus diagnosis without the need for cultivated virus, this would bode well for our ability to generate similar assays for calicivirus and for Norwalk virus.

TYPE SPECIFIC ASTROVIRUS IDENTIFICATION

During the contract year, work was initiated to produce type-specific monoclonal antibodies to each of the five known astrovirus serotypes for type-specific identification of these viruses in immunological and epidemiological studies.

Because we have samples obtained in successive years from the same areas which we have shown contain astroviruses at a relatively high incidence 8% of total diarrheal cases, these samples can be tested with serotype specific astrovirus monoclonal antibodies once they are developed. This will give us information on both prevailing serotypes and possible immunity to them. We have started monoclonal antibody production to astrovirus type 1, which has been found to be the most prevalent type in the limited studies which have been done.

COLLABORATIVE EPIDEMIOLOGICAL STUDIES WITH THE MILITARY

We have continued to perform collaborative studies with U.S. military scientists during the current contract year on the role of viral agents in gastroenteritis.

A series of collaborative efforts with Dr. Peter Echeverria (AFRIMS, Thailand) are the outpatient studies of endemic longitudinally studied diarrhea among children in Bangkok. The adenovirus component has been published (22), and the astrovirus studies are now in press (33). Some of the results are discussed in the section above entitled "Astrovirus etiology of viral gastroenteritis".

Astroviruses have caused outbreaks of gastroenteritis in homes for the elderly, but have not been looked for in younger adult populations. Therefore, we examined for astroviruses in samples obtained from U.S. military personnel with gastroenteritis, in this case from those who were on maneuvers in Honduras, and found that 19.4% (7/36) of swabs were virus positive. Use of convalescent serum in an IEM test on one of the swabs tested showed small, round virus particles which was further evidence that the samples were astrovirus positive. This finding suggests that astroviruses can be important in younger adults as well as in children and the elderly.

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PUBLICATIONS OF WORK SUPPORTED BY THIS CONTRACT

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